The receptor activator of NF- B ligand-osteoprotegerin system in pulpal and periapical disease

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Abstract: AIM: To summarize the collective in vitro, in vivo and clinical evidence of the involvement of the receptor activator of NF- B ligand (RANKL)-osteoprotegerin (OPG) system, a system of two molecules controlling osteoclast differentiation and hard-tissue resorption, in pulpal and periapical pathophysiology. METHODOLOGY: A systematic search related to RANKL and/or OPG and pulp or periapical disease was conducted on Medline, Biosis, Cochrane, Embase and Web of Science databases using keywords and controlled vocabulary. No language restriction was applied. Two independent reviewers first screened titles and abstracts and then the full texts that were initially included. The reference lists of the identified publications were examined for additional titles. RESULTS: A total of 33 papers were identified. In vitro studies (N = 11) revealed that pulpal cells can be stimulated by various inflammatory agents to produce RANKL, whilst many studies did not consider the RANKL/OPG ratio. Animal studies (N = 9) mostly focused on the time course and development of periapical lesions in relation to the RANKL-OPG system. Levels of RANKL and OPG in the necrotizing pulp were not investigated. Human studies (N = 13) showed a steady-state expression of OPG in the odontoblast layer. Conflicting results have been reported regarding the role of RANKL in active apical periodontitis, again because the correlation of this molecule with its inhibitor (OPG) was often disregarded. CONCLUSIONS: There is relatively little information currently available that would highlight the specific role of RANKL and OPG in pulpal and periapical disease. OPG may play a protective role against internal resorption, whilst an increased periapical RANKL/OPG ratio might indicate bone resorption.

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The RANKL-OPG system in pulpal and periapical disease

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Abstract

Aim: To summarize the collective *in vitro*, *in vivo* and clinical evidence of the involvement of the RANKL-OPG system, a system of two molecules controlling osteoclast differentiation and hard tissue resorption, in pulpal and periapical pathophysiology.

Methodology: A systematic search related to RANKL and/or OPG and pulp or periapical disease was conducted on Medline, Biosis, Cochrane, Embase, and Web of Science databases using keywords and controlled vocabulary. No language restriction was applied. Two independent reviewers first screened titles and abstracts and then the full texts that were initially included. The reference lists of the identified publications were examined for additional titles.

Results: A total of 33 papers were identified. *In vitro* studies (N=11) showed that pulpal cells can be stimulated by various inflammatory agents to produce RANKL, whilst many studies did not consider the RANKL/OPG ratio. Animal studies (N=9) mostly focused on the time course and development of periapical lesions in relation to the RANKL-OPG system. Levels of RANKL and OPG in the necrotizing pulp were not investigated. Human studies (N=13) showed a steady-state expression of OPG in the odontoblast layer. Conflicting results have been reported regarding the role of RANKL in active apical periodontitis, again because the correlation of this molecule to its inhibitor (OPG) was often disregarded.

Conclusions: There is relatively little information currently available that would highlight the specific role of RANKL and OPG in pulpal and periapical disease. OPG may play a protective role against internal resorption, whilst an increased periapical RANKL/OPG ratio might indicate bone resorption.

Introduction

The pathogenesis of pulpal and periapical lesions is largely attributed to host inflammatory responses caused by the bacterial infection of the pulp cavity and root canals (Kakehashi *et al.* 1965). Inflammation of the dental pulp due to caries, trauma or iatrogenic causes may sequentially stimulate periapical inflammation. The inflammatory process is initiated in the dental pulp, but may subsequently spread in the periapical region. Persistence of the infection in the pulp space may eventually lead to periapical bone resorption. Clinically this manifests as apical periodontitis, associated with the formation of cysts and granulomas. Histopathologically, apical periodontitis is dominated by dense inflammatory infiltrates and increased osteoclast numbers (Nair 2004). A predominant immunological feature in these conditions is the massive production of inflammatory mediators, dominated by cytokines of the interleukin (IL)-1 family, metabolites of arachidonic acid, such as prostaglandin (PG)E_2, and neuropeptides (Stashenko *et al.* 1998). This is considered as a response of the defending host, attempting to tackle with the gradually establishing infectious microorganisms.

Under physiological conditions, a balance between bone formation and resorption is established at any site of the skeleton. A local switch in this balance towards enhanced bone resorption would result in a bone-related pathology, such as apical periodontitis. When considering the molecular mechanisms that control bone resorption, the state-of-art evidence demonstrates that the process is controlled by the interplay of Receptor Activator of NF-κB Ligand (RANKL) and osteoprotegerin (OPG), belonging to the tumor necrosis factor (TNF) ligand and receptor superfamilies, respectively (Lerner 2004). RANKL was initially identified as a cell membrane-bound ligand responsible for stimulation of osteoclast differentiation and bone resoprtion (Lacey *et al.* 1998, Kong *et al.* 1999). Hence, this molecule came into the early spot-light as the long-sought osteoclast differentiation factor that mediates the cell-to-cell interaction between osteoblasts and osteoclast precursors. It is now known that RANKL is produced either as a membrane-bound or a secreted ligand by osteoblasts, fibroblasts, or

activated T- and B-cells (Lerner 2006). By activating its cognate RANK receptor on the surface of the precursors (essentially cells of the monocyte/ macrophage lineage), it triggers their fusion and differentiation into mature osteoclasts, thus activating bone resorption (Teitelbaum & Ross 2003). On the other hand, OPG is a soluble decoy receptor that blocks RANKL, hence preventing its interaction with RANK and all the down-stream events that lead to osteoclast differentiation (Simonet *et al.* 1997). The production of RANKL and OPG by various cell types is controlled by systemic and local stimuli, including hormones, inflammatory mediators and bacterial products (Lerner 2006, Harokopakis-Hajishengallis 2007). Members of the IL-1 family of cytokines, as well as prostaglandins, are among the most potent inducers of RANKL expression (Liu *et al.* 2010). The overall efficiency of RANKL on osteoclast formation and bone resorption is tightly coupled to the presence and levels of its natural inhibitor OPG. Therefore, it is meaningful to study concomitantly the expression of these two molecules in sites of suspected hard tissue resorption, preferably as their relative RANKL/OPG ratio.

The involvement of the RANKL-OPG system is well established in the pathogenesis of diseases of bone and mineral metabolism, such as rheumatoid arthritis, postmenopausal osteoporosis and bone malignancies (Vega *et al.* 2007), as well as chronic and aggressive marginal periodontitis (Taubman *et al.* 2007, Buduneli & Kinane 2011, Belibasakis & Bostanci 2012). Interestingly, an elevated RANKL/OPG ratio has been demonstrated in the gingival crevicular fluid (GCF) of sites with periodontitis, compared to gingivitis, or healthy sites (Mogi *et al.* 2004, Bostanci *et al.* 2007). An increase of the RANKL/OPG ratio can also be measured in the GCF of teeth subjected to orthodontic toot movement (Nishijima *et al.* 2006). Less conclusive information is available on the involvement of this molecular system in pulpal and periapical pathoses, such as pulpitis and apical periodontitis, or its association with the clinical presentation of these conditions. The objective of this review was to carefully evaluate the involvement of the RANKL-OPG system in the bacterially-induced

inflammatory processes in the pulp and periapical region, by summarizing any collective evidence on *in vitro* models, animal experimentations and clinical studies involving human material. The question of clinical relevance addressed is whether the RANKL-OPG system is differentially regulated in pulpitis and apical periodontitis, and whether this could serve as a measure for differential diagnosis between these conditions, or a prognostic biomarker that can further predict the progression of bone resorption.

Search strategy

The search included all published articles relating RANKL and/or OPG to pulpal and/or periapical disease. Human, animal, and cell culture studies were all considered. The Medline database was searched via the OvidSP search interface with a combined search strategy using keyword search and controlled vocabulary (MeSH-terms) as described in Table 1 (for further details see Supplement 1). The same strategy was adapted and applied using Biosis (OvidSP), the Cochrane library (Wiley), Embase (www.embase.com) and the Web of Science (Thompson). Articles from inception of these databases (Medline 1948, Biosis 1980, Cochrane 1995, Embase 1974, Web of Science 1899) up to and including October 2011 were considered. Publications available in early-online format at the time of the electronic search were included in this review. No language restriction was applied. Furthermore, the reference lists of the identified articles were searched for further titles.

Selection of publications

In a first screening step, the two reviewers (GNB and DKR) independently evaluated titles and abstracts from the electronic search and assessed these with respect to the exclusion criteria applied. Communications were excluded when they were: (a) not related to RANKL or OPG; (b) not related to pulpitis or periapical disease; or (c) mere conference abstracts. Any title included by either reviewer went further to the second screening step, the full-text

evaluation. Both reviewers assessed 40 full texts applying the exclusion criteria mentioned above. Two articles included for full-text evaluation were written in Chinese language. These were translated by a Chinese researcher proficient in English. Disagreement was resolved by discussion and with the help of an experienced referee (MZ).

Search results

Of the 40 full texts initially included, 7 were excluded because they did not meet the search criteria (Fig. 1). Of the 33 included articles, 11 involved laboratory studies on cell cultures, 9 involved animal experimentations, and 13 were observational studies using human clinical samples (Table 2). The following text will review the findings of the identified publications.

Review of identified publications

In vitro studies

The first indication that the RANKL-OPG system may be expressed in the pulp region came with the *in vivo* demonstration that these molecules are immunolocalised on the ameloblast, odontoblast and pulp layers of neonatal murine teeth (Rani & MacDougall 2000). Further *in vitro* investigation demonstrated that both RANKL and OPG are constitutively expressed in murine odontoblasts and dental pulp cells (Rani & MacDougall 2000). Human dental pulp cells are also shown to express RANKL (Uchiyama *et al.* 2009, Belibasakis *et al.* 2011) and OPG (Sakata *et al.* 1999, Mizuno *et al.* 2005, Uchiyama *et al.* 2009, Belibasakis *et al.* 2011) *in vitro.* Nevertheless, in co-culture with appropriate osteoclast precursors, the murine cells failed to induce or even inhibited osteoclast formation (Rani & MacDougall 2000), whereas the human pulp cells supported this action (Uchiyama *et al.* 2009). These findings denote that the control of osteoclastogenesis by dental pulp cells could be species-specific.

Bacteria are putative regulators of RANKL and OPG in dental pulp cells, with potential effect on osteoclast or odontoclast formation. Nevertheless, *in vitro* studies on this topic are very sparse. An *in vitro* biofilm model consisting of six species representative of the supragingival microbiota was investigated for its effects on RANKL and OPG gene expression in human dental pulp and periodontal ligament cells. This multi-bacterial challenge up-regulated RANKL expression in both dental pulp and periodontal ligament cells, but had 4-fold greater effect on the former. Consequently, the relative RANKL/OPG ratio was strongly up-regulated in dental pulp cells, an event which could favour bone or dentine resorption and contribute to the development of apical periodontitis or internal root resorption (Belibasakis *et al.* 2011). *Porphyromonas endodontalis*, a black-pigmented anaerobic species with high association to endodontic infections (van Winkelhoff *et al.* 1992) was investigated for its effects on RANKL in human osteoblasts, rather than dental pulp cells. A time-dependent induction of RANKL protein production was observed, which was evident after 4 h of challenge with *P. endodontalis* (Chen *et al.* 2009). As the regulation of OPG was not investigated, it is not possible to conclude on the net effects on the RANKL/OPG ratio. Experimental systems using human osteoblasts may still be of relevance to apical periodontitis, as endodontic pathogens can well reach the periapical osteoblasts and stimulate the production of bone resorbing factors.

Several inflammatory mediators can also regulate the RANKL-OPG system in dental pulp cells. In an early study, interleukin (IL)-1β and tumour necrosis factor (TNF)-α enhanced OPG expression by human dental pulp cells at a concentration of 0.5 ng/ml, after 12 h of stimulation (Sakata *et al.* 1999). However, this study failed to detect RANKL expression by the cells. These early results would imply a potential role of cytokines on inhibition of dentine or bone resorption, despite their pro-inflammatory capacities. However, later studies have confirmed the expression of RANKL by dental pulp cells and its regulation by pro-inflammatory mediators. Both IL-1α and TNF-α, at concentrations of 10 ng/ml, were shown

to stimulate RANKL gene and protein expression, in a time-dependent manner. Their co-administration had a synergistic effect on RANKL expression, but down-regulated OPG expression (Kim *et al.* 2010). These results in dental pulp cells denote that the relative RANKL/OPG ratio is up-regulated by pro-inflammatory cytokines, well in line with their capacity to stimulate events related to hard tissue resorption. Further on, this work demonstrated that cytosolic phospholipase (cPL)A_2 was responsible for the induction of PGE_2 and the subsequent induction of RANKL expression (Kim *et al.* 2010). Substance P, a sensory neuropeptide, was shown to stimulate PGE_2 and RANKL production by dental pulp cells (Kojima *et al.* 2006). PGE_2 partially mediated the induction of RANKL gene expression in that experimental system, and its subsequent production by the cells. Conditioned medium from these cell cultures stimulated pit formation by osteoclasts on dentine slices. As a mediator of pulpal inflammation (Kvinnsland & Heyeraas 1992), substance P may exert its role in root or periaplical bone resorption by stimulating RANKL in a PGE_2-dependent mechanism. Importantly, the findings of this study imply that dental pulp cell-derived RANKL supports the formation of functional osteoclasts on dentine. Therefore, it is reasonable to speculate that the RANKL-OPG system could control odontoclast formation in the dental pulp, thus mediating internal root resorption. However, this remains to be further elucidated.

A number of biological agents could potentially regulate RANKL and OPG expression in dental pulp cells. As such, inorganic polyphosphate, a biopolymer with several biological functions on eukaryotic cells, has been investigated for its potential effects. Long exposure (3 weeks) of human dental pulp cells to this agent has been shown to induce a 3.3-fold higher OPG expression, compared to the un-stimulated control (Kawazoe *et al.* 2008). Platelet-rich fibrin is a platelet concentrate that resembles a fibrin network and contains a large number of growth factors and cytokines, with the potential to enhance wound healing (Dohan *et al.* 2006). This agent up-regulated OPG production by dental pulp cells, already one day after

stimulation, and persisted over a period of 5 days (Huang *et al.* 2010). Stem cells isolated from human dental pulp have also been considered with regards to OPG expression. These were seeded on silk scaffolds and underwent mechanical tension in a bioreactor environment. OPG expression was slightly increased in cells under tension (Han *et al.* 2010). The induction of OPG in these studies could indicate a protective effect against dentine or bone resorption, and a positive switch of the homeostatic balance towards tissue formation. Nevertheless, RANKL was not studied in this context, and therefore the overall regulation of the RANKL-OPG system cannot be deduced.

In conclusion, little *in vitro* evidence is available on the mechanisms of the regulation of the RANKL-OPG system in dental pulp cells, or in response to endodontic pathogens. Yet, the available information indicates that RANKL and OPG can be regulated by bacterial or pro-inflammatory stimuli in these cells, in a manner that favours bone resorption, and possibly via the mediation of PGE_2. It should be reiterated that future studies in this direction must consider the concomitant investigation of RANKL and OPG. Moreover, relatively limited information is available on the capacity of dental pulp cells to stimulate odontoclast formation via the RANKL-OPG system. This is an open field of study, which could provide answers to the molecular mechanisms governing internal root resorption, and needs to be further pursued.

Animal studies

The first *in vivo* demonstration of the involvement of the RANKL-OPG system in periapical lesions came by immunolocalization studies, in experimental animal models. In particular, periapical localization of RANKL was initially investigated in a rat model whereby the pulp of the mandibular first molars was experimentally exposed (Zhang & Peng 2005a,b). Osteolcast-like cells, which are tartrate-resistant acid phosphatase (or short TRAP)-positive, and RANKL-positive cells were observed periapically, already one week after pulpal exposure. By end of the second week, dense inflammatory infiltrates and periapical bone

resorption was observed, accompanied by a climax in RANKL-positive and osteoclast-like cells. After 4 weeks of exposure, although the chronic inflammatory infiltrates were established, the presence of these osteoclast-like and RANKL-positive cells was reduced to baseline levels, and periapical bone resorption was slowed down (Zhang & Peng 2005a,b). This study revealed the involvement of RANKL in periapical bone resorption, and identified that the peak of its expression is commensurate with a peak of osteoclast activity and bone resorption. This was further confirmed on the mRNA expression level, in a later study using a similar experimental system, which also took into account the expressions of OPG and RANK (Kawashima *et al.* 2007). The periapical expression of RANKL peaked after 2 to 3 weeks of pulpal exposure, and maintained higher than baseline levels, over a period of 8 weeks. RANK and OPG expressions followed a similar kinetic pattern of increase, however not as pronounced as RANKL. The enhancement of OPG expression was explained as a compensatory mechanism for the rapid induction of RANKL. Nevertheless, the relative RANKL/OPG expression ratio peaked over 3 weeks, and persisted at high levels over the 8-week observational period, suggesting an enhanced capacity for bone resorption. Accordingly, expression of pro-inflammatory cytokines IL-1α, IL-1β and TNF-α, with the capacity to induce RANKL, also peaked during the interval between 2 and 3 weeks. Immunohistochemically, RANKL-positive cells with diverse morphologies were detected in the periapical lesion, in close proximity to the alveolar bone and to osteoclast-like cells. These were identified as mainly fibroblastic, but a small proportion of T-cells were also evident. Therefore, this study demonstrated that a local increase in the RANKL/OPG expression ratio, occurring 2 to 3 weeks after pulpal exposure, is concomitant with the expansion of the periapical lesion.

Pulp exposure models in animals allow for the “natural” development of a periapical lesion. Molecules such as RANKL and OPG can thus be studied regarding their role in the development of hard-tissue lesions in real time. However, some of the animal studies

reviewed here may not have taken stringent consideration of the bacterial engagement in this process. In a recent variation of this experimental model, the infectious agent was controlled by co-administration of *Escherichia coli* LPS (soaked on a paper-point) into the exposed distal root canal of the lower first molars, up to the apex (Chuang *et al.* 2012). Periapical bone resorption, expression and localization of RANKL and OPG, and osteoclast formation were followed over a period of 8 weeks. A boost in RANKL expression was observed after 3 weeks of exposure, culminating in 208 % of the unexposed pulp control, after 8 weeks. OPG expression was fluctuating between weeks 1 and 8, but was detected at lower levels than the control group. Accordingly, a very strong induction of the relative RANKL/OPG expression ratio was evident at weeks 7 and 8, which was respectively 30 and 41 times higher than the control group. Increased localization of both RANKL and OPG in the periapical lesions was evident from week 3 onwards, accompanied by an increase in the presence of osteoclast-like cells. This model of LPS-induced periapical lesions in the rat provides insights to the involvement of RANKL and OPG in this infectious process (Chuang *et al.* 2012). Nevertheless, it is difficult to interpret the relative contribution of LPS, due to the lack of a control group where the pulp is exposed, yet not treated with LPS.

Experimental animal models are amenable to technical manipulations, allowing for the study of specific factors in the development of the disease. For instance, ovariectomized rats have been used in order to study the relative effect of estrogen deficiency on the synthesis of RANKL and OPG, in induced periapical lesions (Zhang *et al.* 2007). It was found that in the ovariectomized rat group, more osteoclasts and more RANKL-positive cells were present, than the sham-ovariectomized group, as early as one week after the induction of periapical lesion. Nevertheless, OPG production was also increased at early time points in the estrogen-deficient mice, which by time was reduced to control levels. This is perceived as an early protective response of the tissue, in order to compensate against the overproduction of RANKL and, consequently, bone resorption. Overall, estrogen deficiency appears to

accelerate periapical bone loss, characterised by high local levels of RANKL production (Zhang *et al.* 2007).

Experimental periapical lesion models have also been utilized in genetically modified mice, in order to evaluate the involvement of specific genes in the RANKL or OPG-associated responses. In a mouse model of endodontic infections, the relative role of osteopontin (OPN) was considered (Rittling *et al.* 2010). OPN is a secreted integrin-binding protein that constitutes part of the extracellular matrix of bone. It is also considered to play a functional role in immunoregulatory responses. In this model, periapical tissue RANKL expression was increased already 3 days after infection, and was more than 2-fold higher in the OPN-deficient mice, compared to the wild-type counterparts. After 3 weeks, periapical bone loss was significantly more severe in OPN-deficient mice and was characterized by dense inflammatory infiltrates. This study revealed a potential negative association between OPN and RANKL in endodontic infection, which awaits further investigation (Rittling *et al.* 2010).

In mice lacking the CCR2 chemokine receptor, inoculation of 4 bacterial taxa (*Porphyromonas gingivalis*, *Prevotella nigrescens, Actinomyces viscosus*, *Fusobacterium nucleatum*) into the pulp resulted in larger periapical lesion formation, compared to the wild-type mice. After one week of infection, the periapical expression of RANKL was higher, whereas that of OPG was lower in the CCR2-deficient mice. Nevertheless, these differences were not significant after 2 weeks. This study concluded on a protective role of CCR2 against bacterially-induced periapical osteolysis (Garlet *et al.* 2010).

The relative role of nitric oxide synthase (iNOS) or phagocyte oxidase (PHOX) in a pulp exposure model in mice was also studied (Silva *et al.* 2011). After two weeks, experimental (pulp exposed) iNOS-deficient mice exhibited greater periapical resorption area and number of osteoclasts, and higher RANK, RANKL, but not OPG, expression levels, compared to wild-type mice. On the contrary, PHOX-deficient mice did not exhibit any

periapical osteoclasts and the periapical expressions of RANK, RANKL and OPG were not significantly different, compared to the wild-type mice. Collectively, the results of this study indicate that nitric oxide, but not reactive oxygen species, are involved in the progression of periapical bone resorption (Silva *et al.* 2011). The role of iNOS-inducible nitric oxide in periapical bone resorption has also been demonstrated in an earlier study involving bacterial infection of the root canal with the 4 previously mentioned bacterial species (Fukada *et al.* 2008). In this study, iNOS-deficient mice with exposed pulp exhibited higher number of osteoclasts, and higher RANK, but lower OPG expression, compared to the wild-type experimental mice. Periapical RANKL expression was not different between iNOS deficient and wild-type mice. Hence, iNOS deficiency was considered as a factor causing an imbalance of the bone resorption modulating-factors, and leading to increased infection-induced periapical bone resorption.

In conclusion, the above-described animal studies have mostly involved pulpal exposure models to generate periapical lesions, for the study RANKL and OPG localization and expression. These studies confirm that this molecular system is also implicated in pathological periapical bone resorption, and reveal a locally enhanced RANKL/OPG ratio, in line with observations in other bone-destructive pathoses. However, it is noteworthy that no animal experimentations exist in which the RANKL-OPG system is studied within the dental pulp, in relation to pulpitis. This is an important gap in our knowledge that needs to be fulfilled, as it could reveal mechanisms involved in odontoclast formation and internal root resorption.

Human studies

The systematic search produced 13 publications, which fulfilled the criteria (Table 2). In human teeth, RANKL was first identified in pulps of deciduous molars (Lossdörfer *et al.* 2002). Immunohistochemistry revealed cytoplasmatic granular staining for RANKL in

odontoblasts and pulp fibroblasts in roughly half of the investigated specimens. However, RANKL appears to be expressed at much higher levels in pulps of deciduous teeth undergoing resorption (i.e. shedding) than in counterparts of healthy permanent teeth (Yildirim *et al.* 2008). In healthy deciduous tooth pulps, OPG is expressed at higher levels than RANKL (Yildirim *et al.* 2006). Only one study was identified that investigated the levels of OPG in human pulps of different inflammatory states (Kuntz *et al.* 2001). This investigation revealed intense immunostaining for OPG in the odontoblastic layer of healthy pulps. OPG was not detected in the central area of these pulps. In contrast, in inflamed pulps challenged by gross dental decay, the central aspects showed intense immunostaining also. RANKL levels were not investigated. The steady-state expression of OPG in odontoblasts could constitute a protective response of the pulp against imminent inflammatory dentine resorption.

The remaining 10 publications on RANKL and/or OPG that were identified, dealt with periapical inflammation and its possible modulation. A first immunohistochemical study showed the presence of RANKL in radicular cysts (Tay *et al.* 2004). Immunolocalization of RANKL was in concordance with cells staining for TRAP, i.e. osteoclasts. A subsequent publication confirmed the presence of RANKL at the gene expression level in inflammatory periapical lesions of undisclosed nature (Sabeti *et al.* 2005). RANKL mRNA was also semi-quantified in periapical granulomas, while it was below detection limit in healthy periodontal ligament (Vernal *et al.* 2006). Expression of RANKL on infiltrate leukocytes was further investigated using flow cytometry: monocytes ($CD14^+$) and dendritic cells ($CD83^+$) were the main synthesizers of RANKL in granuloma lesions (Vernal *et al.* 2006). An immunohistochemical study compared RANKL and OPG levels between apical granulomas and radicular cysts (Menezes *et al.* 2006). It was found that the ratio of OPG^+/total cells and that of $RANKL^+$/total cells was higher in granulomas than in cysts. However, the ratio between RANKL and OPG-positive cells did not differ between these two types of periapical

inflammatory lesion. Again, various cell types stained positive for RANKL and OPG, with macrophage-like cells ($CD68^+$) showing the highest intensity. Another investigation compared RANKL (but not OPG) mRNA levels between granulomas and cysts, demonstrating that its expression was significantly higher in granulomas than in cysts (Fukada *et al.* 2009). One study, however, did not identify any difference between total RANKL or OPG protein levels or their ratio between granulomas and radicular cysts (Fan *et al.* 2008).

Few human studies have attempted to investigate the role of the RANKL-OPG system in endodontic disease initiation or progression. In one investigation, periapical lesions were graded according to their inflammatory status (Fan *et al.* 2011). The authors found significantly more RANKL-positive cells in severely inflamed lesions compared to lightly inflamed counterparts. However, the RANKL/OPG ratio was statistically similar between inflammations graded as light, moderate, or intense. The RANKL/OPG ratio at the gene expression levels was also compared between granulomas and periodontal ligament of orthodontically moved teeth (Menezes *et al.* 2008a). Whilst the compression sites of orthodontically moved teeth almost consistently showed RANKL mRNA levels to be higher than OPG counterparts, and tension sites the reversed ratio, i.e. OPG > RANKL, this ratio was inconsistent with granulomas. The upstream transcription of pro-inflammatory cytokine genes regulating bone resorption is modulated by a group of molecules termed suppressors of cytokine signalling (SOCS) (Starr *et al.* 1997). In granulomas, it was shown that RANKL gene expression is negatively correlated to SOCS1 mRNA levels (Menezes *et al.* 2008b).

Only one human study attempted to correlate the presence of infective agents, namely cytomegalovirus and Epstein-Barr virus, to RANKL gene expression in granulomas (Yildirim *et al.* 2006). Whilst there was a higher occurrence of these viruses in the periapical lesions compared to healthy pulp tissues, no correlation was found between their presence and RANKL.

Discussion

This comprehensive review showed that, as of yet, relatively little information is available regarding the specific involvement of the RANKL/OPG system in the progress of pulpal and periapical disease. On the positive side, animal studies have unveiled a relatively clear-cut timeline, during which the RANKL/OPG ratio is up-regulated in the periapical tissues after a microbial challenge from the infected pulp space. However, little is known as to what happens in the pulp space itself. There is also ample space for more *in vitro* and human studies to further understand the molecules under investigation in the context of pulpal and periapical pathophysiology. The following text will discuss these issues according to the type of study. Prior to that, it should be clarified that resorptive processes in cementum and dentine from the periodontal side were not included in the search, as this would have widened the focus of the question beyond the pulpal and/or periapical disease induced by pulpal infection. However, internal resorption was included, because it is clearly related to these infectious processes (Gabor *et al.* 2012).

In vitro studies

It is well known that RANKL and OPG can be expressed by various cell types, and thus, *in vitro* research on pulp cell cultures yields relatively little cutting edge information unless it reveals new insights into the expression, translation, and activation of these molecules. For instance, a point worth investigating is the "shedding" of RANKL from the surface of the dental pulp cells, which could have detrimental effects on dentine resorption, within the confined space of the pulp chamber. In this extent, TNF-α-converting enzyme (TACE) is the metalloproteinase with the highest specificity for cleaving and releasing (i.e. shedding) RANKL from the cell surface into the extracellular environment (Lum *et al.* 1999). TACE production can be stimulated in T-cells by *P. gingivalis* (Bostanci *et al.* 2009), and its GCF levels are elevated in periodontitis and correlate positively with RANKL levels (Bostanci *et*

al. 2008). In human dental pulp cell cultures, it was shown that TACE gene expression is up-regulated in response to challenge with *in vitro* supragingival biofilms (Belibasakis *et al.* 2011). It is therefore worth investigating, in the context of the dental pulp, the shedding of RANKL by TACE or other sheddases, and its subsequent effects on odontoclast formation and dentine resorption.

Apart from the investigation of regulation of the RANKL-OPG system in the endodontic context, it should also be reiterated that more emphasis is to be put on its potential implication in odontoclast formation and dentine resorption during the course of pulpitis, even though this is a more rare pathology. In fact, this is highly feasible if one considers that the standard laboratory model to study osteoclastic "bone" resorption *in vitro* is actually performed on sterile dentine slice preparations, obtained from i.e. from elephant tusks (Tamura *et al.* 1993, Agrawal *et al.* 2012).

Animal studies

In general, the available animal studies considering the RANKL-OPG system have focused on the development of periapical lesions, whilst the study of pulpal disease has been neglected in this respect. The role of the RANKL and OPG within the dental pulp, and their involvement in human pulpitis and pulpal breakdown remains therefore somewhat enigmatic. Considering the regulation of RANKL and OPG during the course of experimental pulpitis may reveal a protective role of OPG against internal dentine resorption. The high levels of OPG in the odontoblast layer of healthy human pulps may suggest a role of this cell zone in the protection of the root canal wall from internal resorption (Kuntz *et al.* 2001). Small internal resorptions are a common sequel to pulpitis (Gabor *et al.* 2012). However, the expression of OPG in odontoblasts may protect the canal wall from clinically manifest internal resorptions, which are extremely rare and appear only to occur in cases of chronic pulpitis, i.e. when the inflammatory stimulus persists without complete tissue breakdown for extended periods of

time (Patel *et al.* 2010). Studies to understand the phenomenon of internal root resorptions in the context of the RANKL-OPG system are definitely warranted and should be performed. In support of this notion, evidence already exists on the participation of this system in the events associated with external root resorption, for instance in the case of orthodontic tooth movement (Low *et al.* 2005, Yamaguchi *et al.* 2006, Tyrovola *et al.* 2010, Nakano *et al.* 2011, Zhou *et al.* 2011).

Regarding the role of RANKL-OPG in periapical tissue remodelling, a most recent study (not identified by the current search because published later) investigated germ-free mice, which were exposed to a known bacterial challenge (Maciel *et al.* 2012). After pulpal exposure of the maxillary first molars, suspensions of *Fusobacterium nucleatum*, or *Peptostreptococcus prevotii*, or mixture of both, were inoculated into the root canals, and followed for up-to 2 weeks. In the mono-infected groups, RANKL expression was similar, whereas in the dual infection group this became significantly reduced after 2 weeks. As that study did not include a non-infected group, it is difficult to evaluate the relative contribution of the two tested species in RANKL induction. However, a potential competitive interaction was revealed, as the co-infection with these two species resulted in reduced RANKL expression. When considering the cells that participate in RANKL-induced periapical bone destruction, the role of T-cells is highlighted (Silva *et al.* 2012). Pulp exposure of the lower first molars in mice resulted in formation of periapical lesion and a remarkable infiltration of CD3-positive T-cells after one week, followed by remission by the end of the second week. More than 50 % of these T-cells were also RANKL-positive. The concomitant presence of endodontic pathogens was also investigated in the pulp exposure / periapical lesion model, by 16S rRNA sequencing. *Pasteurella pneumotropica* and *Enterococcus saccharolyticus* were consistently found in the exposed root canals. The systemic IgG response to *P. pneumotropica* was significantly elevated in the experimental mice group. Moreover, mice immunized with this species exhibited in their lymph nodes T-cells with high antigenic species-specific

responses, including proliferation and RANKL production. Hence, this study indicates that endodontic bacteria colonizing the root canal may induce RANKL expression by activated T-cells, and denotes a pathway by which the adaptive immune responses can induce periapical bone resorption (Silva *et al.* 2012).

Human studies

No human study thus far could clearly correlate the RANKL-OPG system to a clinical condition. Apart from its important role in understanding the molecular mechanisms of hard tissue resorption, this system could prove to be an interesting marker to indicate periapical healing after cleaning and shaping of the root canal system. The advantage of this system is that it is, according to animal studies, clearly related to inflammatory disease progression in the periapical area. The antagonistic action of the two proteins makes the relation of their expression to total protein or other internal controls unnecessary. Instead, the RANKL/OPG ratio can be determined at the protein level, within each sampled lesion. Therefore, when comparing different lesions for the RANKL/OPG ratio, any quantitative variations between sample volumes can be eliminated. However, clinical studies investigating this possibility are currently missing and should be performed.

Conclusion

While much research has been performed on the RANKL-OPG system in other fields such as periodontitis, few studies have addressed questions that could improve our specific understanding of pulpal and periapical disease. In this context, further investigations should elucidate the function of OPG in the odontoblastic layer and its potential role in the protection from internal resorption. Furthermore, clinical studies are warranted to investigate the potential of the RANKL/OPG ratio as an early marker of periapical healing, as well as its association to presence or absence of bacteria, after cleaning and shaping of the root canal

system. Prospective studies measuring the concentrations of RANKL and OPG, as well as their relative ratio, in periapical fluid obtained through the root canal may reveal a differential production pattern between pulpitis and chronic or symptomatic apical periodontitis. This could denote differences in the time-line of periapical bone resorption activity and may confer additional molecular diagnostic value for periapical pathoses.

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Table 1 Example of search strategy (here in Medline database) used in the literature review.

Number	Search history	Results
1	RANK Ligand/ or Osteoprotegerin/	4847
2	("tumor necrosis factor ligand superfamily member 11" or trance protein or "receptor activator of nuclear factor-kappa b ligand" or "receptor activator of nuclear factor kappab ligand" or "receptor activator of nuclear factor kappa b ligand" or "tumor necrosis factor related activation induced cytokine" or "tumor necrosis factor-related activation-induced cytokine" or "osteoclast differentiation factor" or "opgl protein" or "osteoprotegerin ligand" or "rankl protein" or "rank ligand" or "follicular dendritic cell derived receptor 1" or "follicular dendritic cell-derived receptor-1" or "osteoclastogenesis inhibitory factor" or "ocif protein" or "fdcr 1 protein" or "fdcr-1 protein" or osteoprotegerin or tnfsf11 or tnfrsf11b).mp.	5854
3	(OPG and ("gene expression" or "mRNA expression" or "protein expression" or "gingival crevicular fluid" or saliva or serum or plasma)).mp.	1368
4	(trance adj5 (protein or expression or activation or pathway)).mp.	74
5	or/1-4	5925
6	(exp Dental Pulp/ or exp Pulpitis/ or exp Dental Pulp Cavity/ or exp Endodontics/ or (pulp or pulpitis or endodontic or "root canal" or apical or periapical).mp.) pulpitis.mp. or exp Pulpitis/	100050
7	5 and 6	57

Table 2 Classification of all publications included to the review (N=33)

In vitro studies (N=11)	Animal studies (N=9)	Human studies (N=13)
Belibasakis *et al.* 2011	Chuang *et al.* 2011	Fan *et al.* 2008
Chen *et al*. 2009	Fukada *et al*. 2008	Fan *et al.* 2011
Han *et al*. 2010	Garlet *et al.* 2010	Fukada *et al.* 2009
Huang *et al.* 2010	Kawashima *et al*. 2007	Kuntz *et al.* 2006
Kawazoe *et al*. 2008	Rittling *et al*. 2009	Lossdörfer *et al.* 2001
Kim *et al.* 2010	Silva *et al*. 2011	Menezes *et al.* 2006
Kojima *et al.* 2006	Zhang & Peng 2005a	Menezes *et al.* 2008a
Mizuno *et al.* 2005	Zhang & Peng 2005b	Menezes *et al.* 2008b
Rani & MacDougall 2000	Zhang *et al*. 2007	Sabeti *et al.* 2005
Sakata *et al*. 1999		Tay *et al.* 2004
Uchiyama *et al.* 2009		Vernal *et al.* 2006
		Yildrim *et al.* 2006
		Yildrim *et al.* 2008

Figure 1 Flow chart depicting the primary inclusion and subsequent exclusion of articles related to the current topic.